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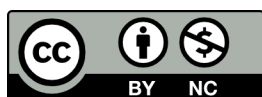
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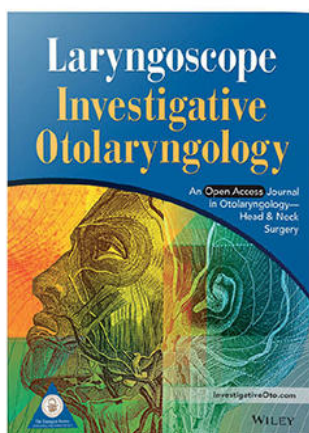


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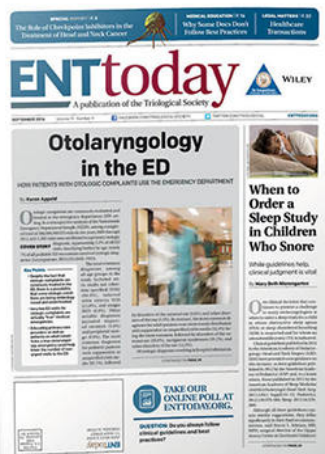


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Development of a Porous Poly(DL-lactic acid-co-glycolic acid)-Based Scaffold for Mastoid Air-Cell Regeneration

Toby W. A. Gould, MSc; John P. Birchall, MD; Ali S. Mallick, MRCS; Tamara Alliston, PhD;
Lawrence R. Lustig, MD; Kevin M. Shakesheff, PhD; Cheryl V. Rahman, PhD

Objectives/Hypothesis: To develop a porous, biodegradable scaffold for mastoid air-cell regeneration.

Study Design: In vitro development of a temperature-sensitive poly(DL-lactic acid-co-glycolic acid)/poly(ethylene glycol) (PLGA/PEG) scaffold tailored for this application.

Methods: Human mastoid bone microstructure and porosity were investigated using micro-computed tomography. PLGA/PEG-alginate scaffolds were developed, and scaffold porosity was assessed. Human bone marrow mesenchymal stem cells (hBM-MSCs) were cultured on the scaffolds in vitro. Scaffolds were loaded with ciprofloxacin, and release of ciprofloxacin over time in vitro was assessed.

Results: Porosity of human mastoid bone was measured at 83% with an average pore size of 1.3 mm. PLGA/PEG-alginate scaffold porosity ranged from 43% to 78% depending on the alginate bead content. The hBM-MSCs proliferate on the scaffolds in vitro, and release of ciprofloxacin from the scaffolds was demonstrated over 7 to 10 weeks.

Conclusions: The PLGA/PEG-alginate scaffolds developed in this study demonstrate similar structural features to human mastoid bone, support cell growth, and display sustained antibiotic release. These scaffolds may be of potential clinical use in mastoid air-cell regeneration. Further in vivo studies to assess the suitability of PLGA/PEG-alginate scaffolds for this application are required.

Key Words: Scaffold, poly(DL-lactic acid-co-glycolic acid), alginate, mastoid, ciprofloxacin.

Level of Evidence: N/A.

Laryngoscope, 123:3156–3161, 2013

INTRODUCTION

The mastoid portion of the temporal bone is composed of interconnected air-filled chambers. This mastoid air-cell system is reported to have a number of important functions including sound conduction, maintaining pressure regulation, gas exchange, material secretion, waste excretion, and middle ear cavity aeration.^{1–3}

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Following mastoidectomy, mastoid obliteration procedures are focused on filling the mastoid cavity and not on repair of the mastoid bone structure. However, the only way to restore the vital functions of the mastoid is to fully regenerate the air cells within the bone.

Various polymers have been investigated for use as scaffolds in bone regeneration, including poly(DL-lactic acid-co-glycolic acid) (PLGA), which has a long history of use as a degradable implant material.⁴ As previously described by Dhillon et al., blending PLGA with a plasticizer such as poly(ethylene glycol) (PEG) allows the production of temperature-sensitive particles with a reduced glass transition temperature (T_g) of 37°C.⁵ When these particles are mixed with a carrier solution at room temperature, a paste is formed that can be molded into any size or shape. The paste then hardens into a solid scaffold at 37°C due to temperature-induced PEG leaching. The ability of this scaffold system to regenerate bone in vivo has recently been demonstrated in a mouse calvarial defect model.⁶

We describe the modification of this scaffold technology for potential use in mastoid air-cell regeneration. A key advantage is the ability to paste the material into a cavity of any size or shape followed by scaffold solidification at body temperature. To optimize the scaffold structure, we describe the inclusion of alginate beads in the formulation to act as a porogen, creating scaffolds with a high level of macroporosity. We also investigate in vitro release of ciprofloxacin from the scaffolds with the intention of providing local, sustained antibiotic delivery to decrease postoperative infections or continuation of underlying infections of the middle ear.

MATERIALS AND METHODS

Mastoid Bone Sample Preparation

A section of mastoid bone approximately 3×3 cm was excised from fresh-frozen human cadaveric temporal bone.

PLGA/PEG Particle Production

Particles were fabricated from blends of PLGA 85:15 DLG 4CA, 53 kDa (Lakeshore Biomaterials, Birmingham, AL) and PEG 400 (Sigma-Aldrich, Poole, UK) as previously described by Dhillon et al.⁵ Briefly, a mixture of 93.5%:6.5% PLGA:PEG (wt/vol) was blended at 80°C to 90°C, mixed, and allowed to cool. Cooled polymer was ground into particles and sieved to obtain the 100- to 200- μ m size fraction.

Alginate Bead Fabrication

A solution of 1% (wt/vol) sodium alginate (Acros Organics, Loughborough, UK) was prepared in phosphate-buffered saline (PBS). A total of 10 mL of alginate solution was loaded into a syringe and ejected from a 23-gauge needle into a gently agitated bath of 25 mL 5% (wt/vol) calcium chloride (Sigma-Aldrich). The beads were strained using a 100 μ m sieve and rinsed with deionized H₂O.

Scaffold Preparation

Triplicate scaffolds were prepared in polytetrafluoroethylene molds producing cylindrical scaffolds 12 mm in length and 6 mm in diameter. PLGA/PEG particles were mixed with saline and varying alginate bead masses to give a range of weight percentages from 0% to 80% alginate. Ratio of PLGA/PEG particles to saline was 1:0.6 (particles:saline). Particle paste was packed into the mold, which was placed at 37°C for 4 hours to allow scaffold formation. For cell seeding experiments, scaffolds were UV sterilized for 80 minutes.

Ciprofloxacin-loaded scaffolds were prepared for drug-release assays using two different methods, A and B. In method A, ciprofloxacin solution (Sigma-Aldrich) was mixed with the PLGA/PEG particles and alginate beads to produce a paste, which was used to prepare scaffolds as described previously (100 μ g ciprofloxacin per scaffold). In method B, ciprofloxacin was added into the PLGA/PEG melt-blend at 80°C to 90°C on a hotplate. The ciprofloxacin-PLGA/PEG particles were then fabricated and used to produce scaffolds as described (100 μ g ciprofloxacin per scaffold).

Porosity Measurements

Triplicate scaffolds prepared using 100% PLGA/PEG, 80% PLGA/PEG-20% alginate, 60% PLGA/PEG-40% alginate, 40% PLGA/PEG-60% alginate and 20% PLGA/PEG-80% alginate were sintered for 4 hours at 37°C followed by freeze-drying for 24 hours, causing the alginate beads to dehydrate. The porosity of the scaffolds was calculated using the bulk density (BD) and particle density (PD) values as follows: $(1 - \text{BD})/(\text{PD} \times 100) = \% \text{ Porosity}$.

X-ray Micro-Computed Tomography

High-resolution three-dimensional (3D) images of the mastoid bone and scaffold samples were acquired using a laboratory x-ray micro-computed tomography (CT) system (μ CT-40; Scanco Medical AG, Brüttisellen, Switzerland). The reconstructed field of view was 12.3 mm for the scaffold specimens and 30.7 mm for the mastoid bone specimen, with isotropic

nominal resolutions of 12 and 15 μ m, respectively. Approximately 1 cm in length was scanned, spanning 800 slices (scaffolds) or 667 slices (bone). The bone or scaffold structure was binarized using a visually selected fixed global threshold. The surface of the 3D structure was triangulated and displayed using the manufacturer's software (μ CT Ray v3.8; Scanco Medical AG).

Mechanical Properties

PLGA/PEG particles sinter over time at 37°C to form scaffolds. Assessing the mechanical properties of the scaffolds over time at 37°C reveals if solidification due to particle sintering has occurred. Triplicate scaffolds prepared using 40% PLGA/PEG-60% alginate were sintered at 37°C for 3, 4, or 5 hours. Compressive strength was tested using a TA.HDPlus texture analyzer (Stable Microsystems, Surrey, UK).

Scanning Electron Microscopy

Scaffolds were mounted on aluminum stubs and sputter-coated with gold at an argon current rate of 30 mA for 3 minutes. Scaffold structural morphology was examined using a scanning electron microscope (Jeol, Herts, UK; model JSM-6060LV) at 10 kV.

Human Bone Marrow Mesenchymal Stem Cell Culture

Human bone marrow mesenchymal stem cells (hBM-MSCs) were cultured in mesenchymal stem cell media (TCS Cell Works, Buckingham, UK) supplemented with 5% fetal calf serum, 1% mesenchymal stem cell growth supplement, 1% penicillin/streptomycin solution (all TCS Cell Works), 1% L-glutamine (Gibco, Paisley, UK) (200 mM), and 1% nonessential amino acids (Sigma-Aldrich). Cells were maintained in a humidified tissue-culture incubator at 37°C and with 5% CO₂.

Cell Seeding on Scaffolds

Triplicate scaffolds for both test and control groups were pre-wet with 100 μ L media and placed in the incubator at 37°C for 1 hour. Then 2×10^5 cells in a 30 μ L suspension was seeded per scaffold. The scaffolds were incubated for 2 hours at 37°C before the addition of 1 mL media to each well.

Prestoblue Metabolic Activity Assay

The Prestoblue (Invitrogen Life Sciences, Paisley, UK) metabolic activity assay was performed on triplicate scaffolds 1, 2, 3, 5, and 7 days postseeding. Each scaffold was submerged in 1 mL of media. A total of 111 μ L of Prestoblue was added to each well, and the scaffolds were incubated at 37°C for 25 minutes. Triplicate 100 μ L media samples from each well were read on a Tecan plate reader with the excitation wavelength set to 535 nm and the emission wavelength set at 615 nm.

Live/Dead Staining

Live/dead staining was performed on triplicate scaffolds 3 days postseeding. A staining solution was prepared in PBS containing 20 μ g mL⁻¹ propidium iodide (Sigma-Aldrich) and 1 μ g mL⁻¹ fluorescein diacetate (Calbiochem, Watford, UK). Then 1 mL of the staining solution was added to each scaffold and incubated at room temperature for 15 minutes before visualization using a Leica LCS confocal microscope. Live cells stain green and dead cells stain red.

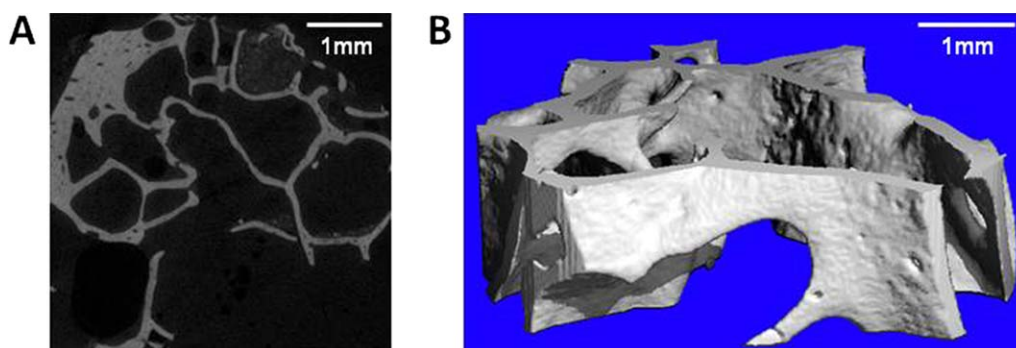


Fig. 1. Microstructure of human mastoid bone. (A) Micro-computed tomography (CT) x-ray slice image of human mastoid bone. (B) Micro-CT three-dimensional reconstruction of human mastoid bone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Ciprofloxacin Release Assay

Triplicate scaffolds loaded with 100 μ g ciprofloxacin prepared using method A or B as described previously were placed in 3 mL PBS and incubated at 37°C. At various time intervals, the PBS was replaced with 3 mL fresh PBS, and 100 μ L of PBS containing released drug was sampled in triplicate at 315 nm on a plate reader (Tecan, Reading, UK) and concentration of drug measured. Non-drug loaded scaffolds containing only PBS were used as a control for background

absorbance values. Data are presented as cumulative drug release as a function of time.

RESULTS

Microstructure of Human Mastoid Bone

Human mastoid bone microstructure was analyzed using micro-CT. The porosity of the bone was 83%. The

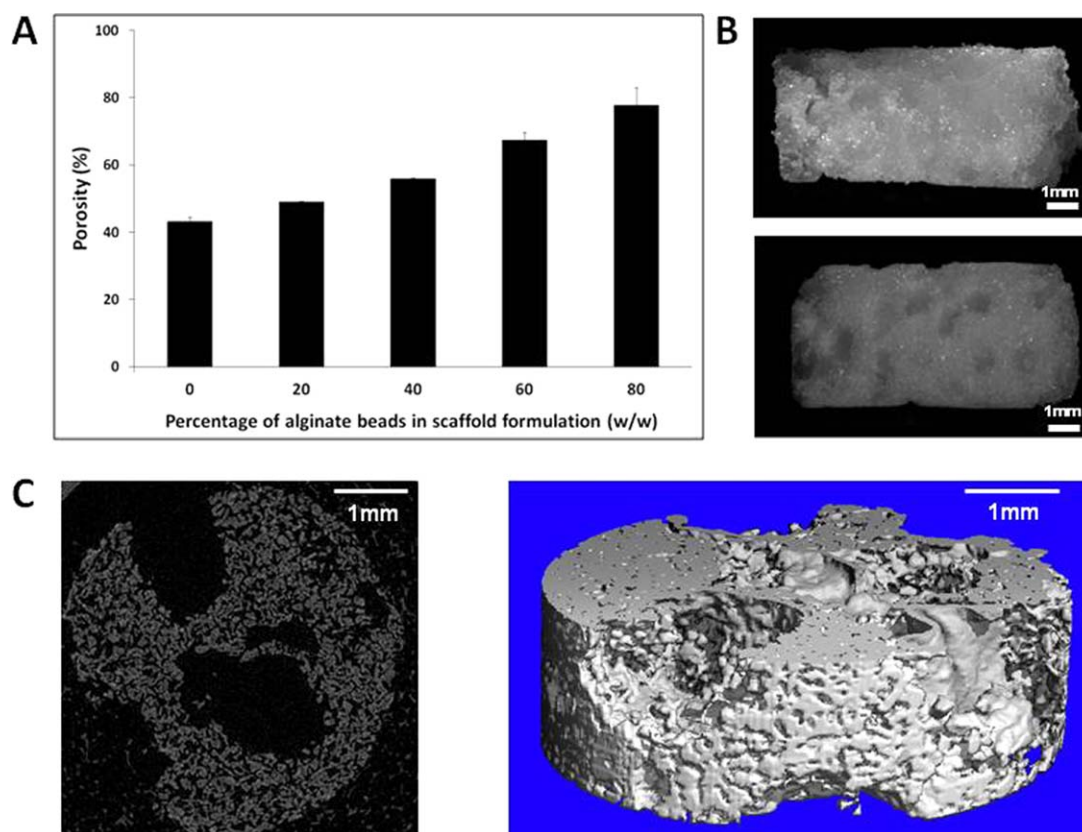


Fig. 2. Microstructure of poly(DL-lactic acid-co-glycolic acid) (PLGA)/poly(ethylene glycol) (PEG)-alginate scaffolds. (A) Porosity of PLGA/PEG-alginate scaffolds calculated by density measurements. (B) Light microscope images of 40% PLGA/PEG-60% alginate scaffolds before freeze-drying (top image) and after freeze-drying for 24 hours (bottom image). (C) Micro-computed tomography x-ray slice and three-dimensional reconstruction of 40% PLGA/PEG-60% alginate scaffold after freeze-drying for 24 hours [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

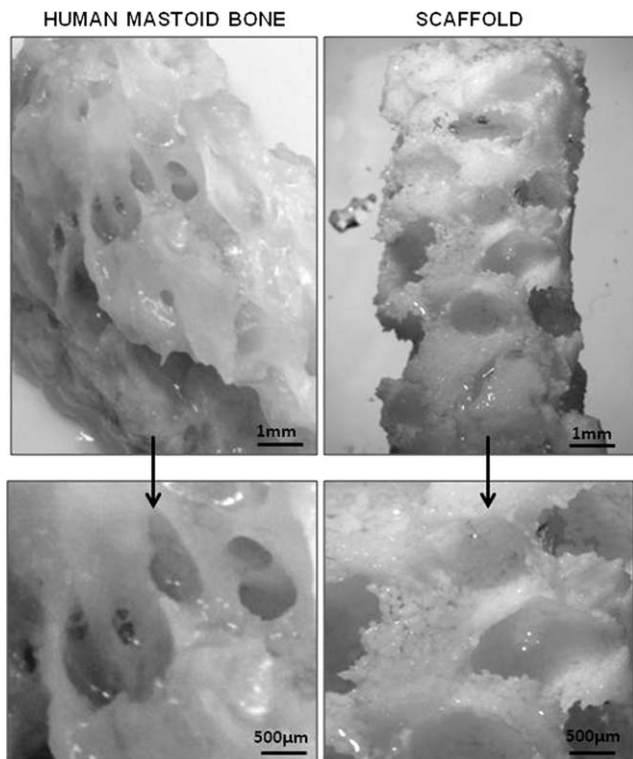


Fig. 3. Image comparison of human mastoid bone and poly(DL-lactic acid-co-glycolic acid) (PLGA)/poly(ethylene glycol) (PEG) scaffold. Light microscope image of human mastoid bone section (left) and 40% PLGA/PEG-60% alginate scaffold (right). Scaffold was submerged in saline at 37°C for 2 weeks before imaging.

physiology of the bone was observed in both x-ray images and 3D reconstruction (Fig. 1). Pores were observed throughout the structure with sizes averaging 1.3 mm, as measured by micro-CT.

Microstructure of PLGA/PEG-Alginate Composite Scaffolds

Scaffolds containing different amounts of alginate beads were sintered for 4 hours. At 37°C, alginate degrades in 3 to 4 days, whereas PLGA/PEG particles degrade in 2 to 3 months. Therefore the alginate beads will degrade rapidly in vivo, leaving a highly porous PLGA/PEG scaffold structure. To mimic this degradation effect, the scaffolds were freeze-dried for 24 hours before porosity measurements to dehydrate the alginate beads. Porosity was assessed by density measurements and increased from 43% (100% PLGA/PEG-0% alginate) to 78% (20% PLGA/PEG-80% alginate) (Fig. 2A). The 20% PLGA/PEG-80% alginate scaffolds were fragile when handled during cell culture experiments, therefore 40% PLGA/PEG-60% alginate scaffolds were used in all subsequent experiments (herein referred to as PLGA/PEG-alginate scaffolds).

Light microscope images of PLGA/PEG-alginate scaffolds were taken before and after freeze-drying (Fig. 2B). The residual pores created by the dehydrated alginate beads can be seen in the image after freeze-drying and in the micro-CT images in Figure 2C. PLGA/PEG-

alginate scaffolds were submerged in saline for 2 weeks at 37°C to visually compare the physical structure of mastoid bone with the scaffolds following alginate bead degradation at this temperature (Fig. 3).

Particle Sintering Within PLGA/PEG-Alginate Scaffolds

PLGA/PEG-alginate scaffolds were sintered for 3, 4, and 5 hours at 37°C. Compressive strength tests were performed to assess the ability of the PLGA/PEG particles to sinter in the presence of alginate beads (Fig. 4). Following 3 hours at 37°C, the compressive strength values were 20 kPa on average. This increased over the next hour at 37°C to 0.2 MPa and further increased to 0.38 MPa by 5 hours. The microstructure of a scaffold sintered for 4 hours at 37°C and freeze-dried for 24 hours is shown in the scanning electron microscopy image in Figure 4B.

Cell Proliferation on PLGA/PEG-Alginate Scaffolds

The hBM-MSCs were seeded onto the scaffolds, and the Prestoblu cell viability assay was performed over 7

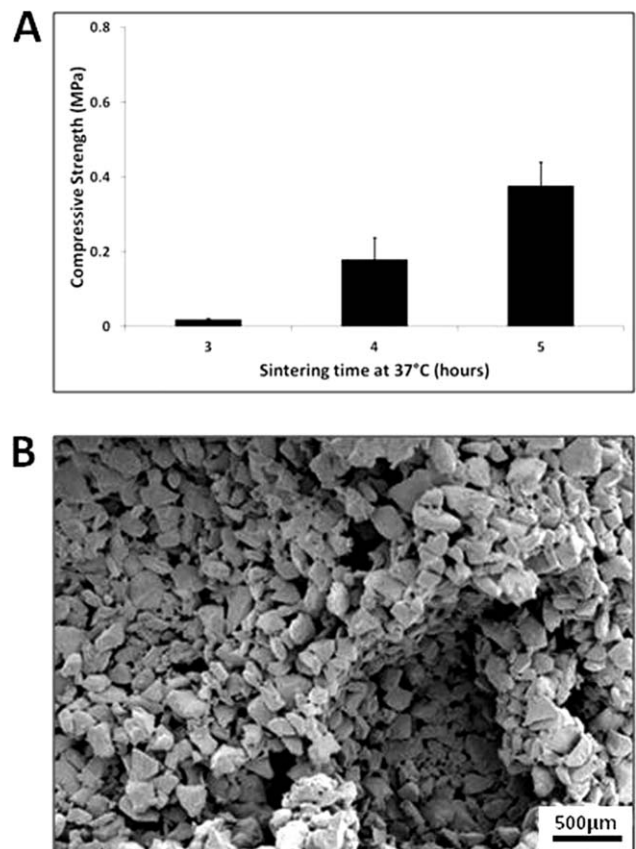


Fig. 4. Poly(DL-lactic acid-co-glycolic acid) (PLGA)/poly(ethylene glycol) (PEG) particle sintering within PLGA/PEG-alginate scaffolds at 37°C. (A) Compressive strength of 40% PLGA/PEG-60% alginate scaffolds sintered for 3, 4, and 5 hours at 37°C. Error bars represent standard deviation. (B) Representative scanning electron microscopy image of a 40% PLGA/PEG-60% alginate scaffold sintered for 4 hours at 37°C and freeze-dried for 24 hours.

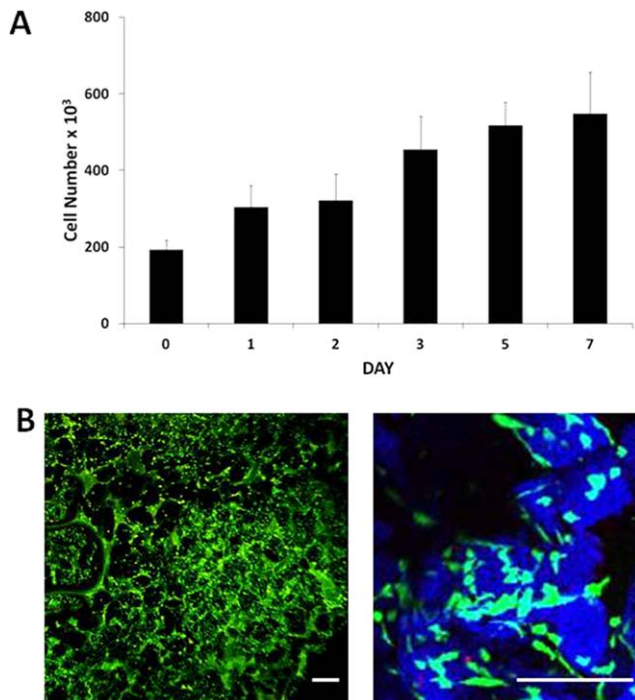


Fig. 5. Proliferation of human bone marrow-derived mesenchymal stem cells on poly(DL-lactic acid-co-glycolic acid) (PLGA)/poly(ethylene glycol) (PEG)-alginate scaffolds. (A) Number of viable human bone marrow mesenchymal stem cells (hBM-MSCs) on 40% PLGA/PEG-60% alginate as measured by the Prestobblue metabolic activity assay over 7 days in culture. Error bars represent standard deviation. (B) Representative images of hBM-MSC-seeded 40% PLGA/PEG-60% alginate scaffold 3 days postseeding following live/dead staining. PLGA/PEG particles rendered in blue using Leica LCS confocal macroscope software to visualize the particles (blue) and pore spaces between particles (black). Size bar = 200 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

days (Fig. 5A). The cell number increased during this time from 2×10^5 cells per scaffold to 6.8×10^5 cells per scaffold, demonstrating hBM-MSC proliferation. Scaffolds were subjected to live/dead staining 3 days after cell seeding to visualize viable (green) and nonviable (red) hBM-MSCs within the scaffolds (Fig. 5B).

Antibiotic Release From PLGA/PEG-Alginate Scaffolds

Ciprofloxacin-loaded scaffolds were prepared as described previously using two different loading methods for scaffold preparation, A and B. In method A, ciprofloxacin solution was mixed with the PLGA/PEG particles and alginate beads to produce a paste, which was used to prepare scaffolds as described (100 μ g ciprofloxacin per scaffold). In method B, the appropriate amount of ciprofloxacin was added into the PLGA/PEG melt-blend. High burst release of ciprofloxacin after 24 hours (73%) was observed with the scaffolds prepared using method A (Fig. 6). Burst release was minimized to 30% using method B, where the drug was mixed within the PLGA/PEG at the melt-blend phase. Ciprofloxacin release from scaffolds prepared using method A slowed down to an average of 0.1% drug release per day from day 7 until

day 53 when release stopped at a total of 97% ciprofloxacin released.

DISCUSSION

An ideal material for mastoid air-cell regeneration has not yet been identified, therefore alternatives have been investigated in recent years including biphasic calcium phosphate granules mixed with fibrin sealant and polycaprolactone-tricalcium phosphate composites.^{7,8} These materials contain ceramic components that may have similar disadvantages to hydroxyapatite for this application, such as extrusion and infection. Bioactive glasses such as BonAlive (BonAlive Biomaterials, Turku, Finland) have recently been reported to show promise in mastoid bone repair procedures; however, the glass granules occasionally leak into the ear canal.⁹ Biodegradable materials have also been assessed including cell-loaded hyaluronic acid gel and growth factor-loaded collagen implants.^{10,11} These materials show promise for bone repair, but they lack the macroporosity required to regenerate the highly porous structure of the mastoid air-cell system.

Here we describe the development of a biodegradable polymer scaffold tailored for mastoid air-cell regeneration. The scaffold is based on PLGA/PEG particle paste, which can be molded into any size or shape and hardens into a solid scaffold at 37°C.⁵ To our knowledge, this is the first description of a biodegradable particulate polymer scaffold that can be pasted into a cavity and that hardens in vivo for this application. The ability to paste the scaffold around the mastoid cavity offers improved clinical application and tissue approximation when compared to implants.

To aid scaffold design it is important to take into account the structure of the mastoid air cells. Therefore mastoid bone was visualized using light microscopy and

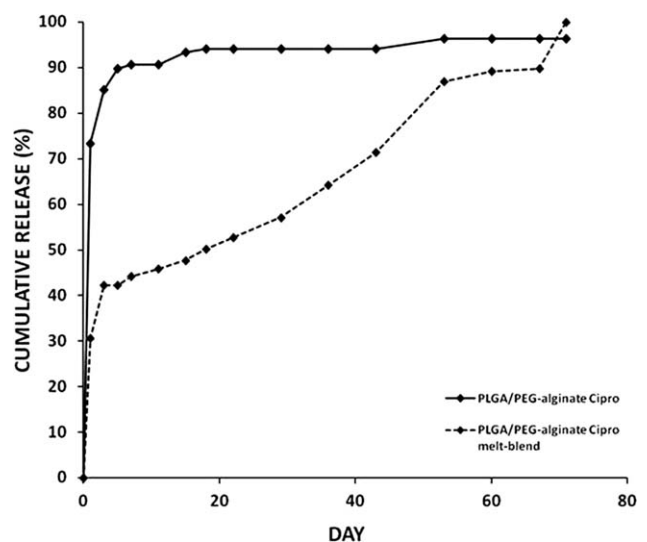


Fig. 6. Ciprofloxacin release from poly(DL-lactic acid-co-glycolic acid) (PLGA)/poly(ethylene glycol) (PEG)-alginate scaffolds. Cumulative release profiles for ciprofloxacin from 40% PLGA/PEG-60% alginate scaffolds represented as percentage of total loaded drug released. Two different drug-loading methods were used, method A (PLGA/PEG-alginate cipro) and method B (PLGA/PEG-alginate cipro melt-blend).

micro-CT. The overall porosity of the bone was measured at 83% with an average pore diameter of 1.3 mm. Our intention was to optimize scaffold structure with regard to generating large air cells mimicking those observed in mastoid bone. Alginate beads of 1- to 1.5-mm diameter were successfully used as porogens to create pores in the PLGA/PEG scaffolds. Scaffold porosity was enhanced from 43% to 78% with increasing amount of alginate beads.

In the current study, the mastoid bone sample displayed a porosity level of 83%. The scaffold formulation with a porosity value closest to that of the mastoid bone sample was 20% PLGA/PEG-80% alginate, at 78%. Because of the fact that scaffolds produced using this formulation were fragile when handled during cell culture experiments, 40% PLGA/PEG-60% alginate scaffolds (64% porous) were used in all subsequent experiments. It will be important to assess the ability of both formulations to regenerate mastoid air cells in future in vivo studies to determine the best formulation to take forward for clinical use.

The ability of the scaffold to harden at body temperature is an important property, as it ensures the polymer is retained where it is applied. This hardening process could potentially allow the polymer to act as an obliteration material in addition to promoting air-cell regeneration within the mastoid bone. As the formulation contained 60% alginate beads, it was necessary to assess the ability of the PLGA/PEG particles to sinter in this formulation. Scaffold hardening at 37°C was demonstrated by an increase in scaffold compressive strength over time, confirming the ability of the PLGA/PEG particles to fuse into a solid scaffold in the presence of the alginate beads.

PLGA/PEG scaffolds are capable of regenerating bone in vivo. The formulation used in the current study contained 40% PLGA/PEG and 60% alginate; therefore the ability of hBM-MSCs to grow on the scaffolds in vitro was assessed. Our results demonstrate attachment and proliferation of hBM-MSCs on the scaffolds over a 7-day period, demonstrating the potential for cells to proliferate on the scaffolds, which has implications for their successful use for bone repair in vivo.

To reduce the likelihood of secondary complications arising from postoperative infection or recurrence of underlying middle ear infections, we incorporated ciprofloxacin into the PLGA/PEG-alginate scaffold formulation as it is commonly used in the treatment of middle ear infections. We used two different antibiotic loading methods. Method A involved mixing the drug into the PLGA/PEG and alginate bead paste before scaffold formation, trapping the drug inside the scaffold during solidification at 37°C. Method B involved mixing the drug into the PLGA/PEG at the melt-blend phase, therefore loading it inside the particles. Incorporation of ciprofloxacin into polymer melt-blends at high temperature (80°C–150°C) has been reported using polycaprolactone/poloxamine, with the antibiotic being shown to retain its antibacterial activity following exposure to these high

temperatures.¹² In our method, the ciprofloxacin was heated to 80°C. A high burst release of 73% ciprofloxacin after 24 hours was observed with the scaffolds prepared using method A, caused by drug on the surface of the particles releasing immediately. This burst release was minimized to 30% using method B, as the drug is trapped inside the particles, therefore reducing the amount of drug available for immediate release. Scaffolds produced using method A released for 7 weeks, with a daily release rate of approximately 0.1% per day. Scaffolds produced using method B generated an improved release profile with a lower burst release phase followed by 10 weeks release of approximately 1% per day.

CONCLUSION

We describe the development of a porous, biodegradable PLGA/PEG-alginate scaffold that can be pasted into a cavity of any size or shape. Scaffold porosity can be controlled by varying the amount of alginate beads in the formulation, with a maximum of porosity of 78% achieved. The scaffolds support the growth of hBM-MSCs and display sustained release of ciprofloxacin for 7 to 10 weeks in vitro. Future studies will focus on the assessment of this scaffold formulation for mastoid air-cell regeneration in vivo.

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